

Polypeptide

The present invention relates to the specific lipid binding ability of the PH domain of Ect 2. In particular, the invention relates to the identification of this ability and the use of this domain in assays for identifying modulators of Ect 2 activity.

BACKGROUND TO THE INVENTION

Proliferative growth of normal cells requires an orderly progression through a series of distinct steps, a process known as the cell cycle. Progression through the cell cycle is modulated by nutrient availability, cell size and growth factors through complex signalling pathways involving phosphorylation cascades and the strictly regulated expression and stability of specific proteins required at each phase of the cell cycle.

The phases of the cell cycle begin with the M phase, where cytoplasmic division (cytokinesis) occurs. The M phase is followed by the G1 phase, in which the cells resume a high rate of biosynthesis and growth. The S phase begins with DNA synthesis, and ends when the DNA content has doubled. The cell then enters the G2 phase, which ends when mitosis starts, signalled by the appearance of condensed chromosomes. Terminally differentiated cells are arrested in the G1 phase, and no longer undergo cell division.

The sequence of cell cycle events is rigorously controlled at specific checkpoints to ensure that each discrete stage in the cell cycle has been completed before the next is initiated. Human diseases associated with abnormal cell proliferation, including cancer, result when these rigorous controls on cell cycle progression are perturbed.

The elucidation of the signalling pathways involved in the cell cycle and their specific role in its control will provide novel opportunities in the prophylactic, diagnostic and therapeutic management of cancer and other proliferation-related diseases (e.g., atherosclerosis).

On the other hand, it is also sometimes desirable to enhance proliferation of cells in a controlled manner. For example, proliferation of cells is useful in wound healing and where growth of tissue is desirable. Thus, identifying signalling pathways and mechanisms along with modulators which promote, enhance or deter the inhibition of proliferation is desirable.

Despite the desirability of identifying cell cycle components and modulators, there is a deficit of such compounds in the field. One approach to identifying new compounds is to elucidate protein signalling interactions and to identify compounds which inhibit or enhance these interactions.

The superfamily of small (21 kDa) GTP binding proteins (small G proteins) comprises 5 subfamilies: Ras, Rho, ADP ribosylation factors (ARFs), Rab, and Ran, which act as molecular switches to regulate numerous cellular responses. Members of the Rho family of GTPases, include RhoA, -B, and -C, Rac-1 and -2, and Cdc42.

Rho-family small GTPases can be found in both GDP and GTP bound states. When bound to GTP they interact with downstream members of signalling cascades that can ultimately lead to changes in a wide variety of cellular processes including regulation of gene expression, regulation of the actin cytoskeleton and cell adhesion, regulation of smooth muscle contraction, cell morphology, cell motility, neurite retraction, cytokinesis, and cell transformation (Hall, A. Science (1998) 279:509-514).

The interchange between GTP and GDP bound states is under the control of positive and negative regulatory mechanisms. GTPase activator proteins (GAPs) increase GTPase activity thus promoting the GDP bound state that in turn can be sequestered and stabilised by an interaction with a GDP dissociation inhibitor (GDI). Both GAPs and GDIs function to decrease the levels of GTP bound Rho-family proteins thus turning signalling "off". In contrast Guanine-nucleotide exchange factors (GEFs) interact with the small GTPase and promote the exchange of GDP for GTP, thus enhancing signalling in the pathway.

Ect2 is a member of the Rho-GEF family of proteins and as such promotes the exchange of GDP for GTP on the Rho-family of GTPases (Rho, Rac and cdc42) (Tatsumoto *et al*, 1999). The *ect2* gene is conserved at the sequence and functional levels in mammals and insects. The *pebble* gene in *Drosophila* GenBank ID # (GI) 5817603) is the orthologue of mouse (GI293331) and human *ect2*, and is required for initiation of cytokinesis (Lehner CF, J. Cell Sci. (1992) 103: 1021-1030; Prokopenko SN, et al., Genes Dev (1999) 13(1 7):2301-2314).

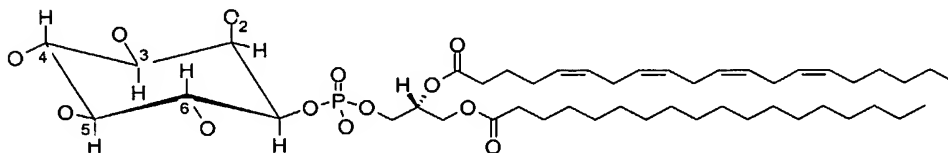
The protein is 883 amino acids long and has a molecular weight of approximately 100 kD. It consists of 4 functional domains, BRCT domains (aa 140 - 229 and 235 – 323), a DbpA-homology (DH) domain (aa 421 – 610) and a Pleckstrin-homology domain (aa 644 – 763). The tandem DH-PH domain construction is typical of GEFs. Data has shown that the DH domain interacts with and promotes nucleotide exchange on the partner GTPase and that the PH domain is involved in targeting or regulation of DH exchange activity (reviewed by Zheng *et al.*, 2001).

Ect2 phosphorylation, which is required for its exchange activity, occurs during G2 and M phases. Human Ect2 is involved in the regulation of cytokinesis. The human *ect2* gene is located on the long arm of chromosome 3, at 3q26 (Takai S. et al., Genomics (1995) 27(1):220-222), a region of increased copy number and expression in a large number of cancers (Bitter MA, et al., Blood (1985) 66(6): 1362-1370; Kim DH, et al., Int J Cancer. (1995) 60(6):812-8 19; Brzoska PM, et al., Cancer Res. (1995) 55(14):3055-3059; Balsara BR, et al., Cancer Res. (1997) 57(1 1):2116-2120; Heselmeyer K. et al., Genes Chromosomes Cancer (1997) 19(4):233-240; Sonoda G, et al., Genes Chromosomes Cancer. (1997) 20(4):320-8). Data available from the National Cancer Institute (www.ncbi.nlm.nih.gov/ncicgap) indicates that human *ect2* is overexpressed in cancers of the ovary, uterus, parathyroid, testis, brain, and colon.

A number of domains having homology to known functional domains have been identified in the Ect 2 polypeptide. However, their function to date remains unclear. The role of these domains and the possibility of modification of Ect 2 activity through

modifying these domains remains an important target for modifying cell cycle progression.

- Prior to stimulation by an upstream signalling event many GEFs are present in a partially active or even inactive state. Activation can result from phosphorylation events such as those on Vav where phosphorylation on Y174 causes the unfolding of an α -helix shielding the Rac interaction site thus allowing the exchange reaction to proceed (Bustelo, 2000). Ect2 itself has been reported to show maximal exchange activity following a phosphorylation event during mitosis (Tatsumoto *et al.*, 1999).
- Protein:protein interactions can also affect GEF activity as evidenced by the interaction between the armadillo domain of APC and the Rac-specific GEF Asef (Kawasaki *et al.*, 2000). This interaction led to enhanced Asef GEF activity and ultimately led to altered cell morphology in MDCK cells.
- Phosphatidylinositol (PtdIns) (shown below) is the core unit of the eukaryotic inositol lipids. It consists of D-myo-inositol-1-phosphate linked to diacylglycerol via a phosphodiester bond. The inositol head group has 5 free hydroxyl groups of which those at positions 3,4 and 5 have to date been identified as phosphorylated either singly or in combination the cellular environment.



- Phosphatidylinositol and its phosphorylated derivatives are collectively referred to as phosphoinositides or PIs. They are present in all membranes and have been shown to be substrates for a variety of cellular enzymes including kinases, lipases and phosphatases.

Phosphatidylinositol is the most abundant of the inositol lipids found in mammalian cells present at concentrations 10 – 20 times those of PtdIns4P and PtdIns4,5P₂ in normal resting cells. Of singly phosphorylated PtdIns approaching 95 % is present as PtdIns4P, with the remainder consisting of PtdIns3P and PtdIns5P. PtdIns4,5P₂ is by far the most abundant doubly phosphorylated species consisting of over 99 % of such lipids with PtdIns3,4P₂ and PtdIns3,5P₂ forming the remainder. Levels of the triple phosphorylated PtdIns3,4,5P₃ (Stephens *et al.*, 2000; Rameh *et al.*, 1997)

Binding of phospholipids to the PH domain of Dbl family GEFs can also lead to modulation of GEF activity. Vav and Sos GEF activities have been shown to be activated by binding of PtdIns3,4,5P₃ (Han *et al.*, 1998, Nimnual *et al.*, 1998) and to be inhibited by interaction with PtdIns4,5P₂ (Das *et al.*, 2000). The proto-oncogene Dbl has shown decreased GEF activity interacting with both PtdIns4,5P₂ and PtdIns3,4,5P₃ (Russo *et al.*, 2001)

Phospholipid binding is mediated by the Pleckstrin homology domain of Ect2 (aa 644 – 763). The small functional domain, present in many proteins involved in signal transduction, was originally identified in pleckstrin, a substrate of protein kinase C. The domain fold, 2 perpendicular anti-parallel beta sheets with a c-terminal amphipathic helix, is structurally very similar to phosphotyrosine binding fold (PTB), the Enabled/VASP homology domain (EVH1) and the Ran-binding domain (RanBD) although there is little similarity at the amino acid level.

Lipid binding by PH domains has been investigated by a numbers of investigators and this has led to the classification of PH domains by lipid binding specificity. Lipid binding PH domains can generally be placed into one of four categories shown below (Kavran *et al.*, 1998, Rameh *et al.*, 1997):

	Lipid Specificity	Example
Group I	PtdIns4,5P ₂ , Ins1,4,5P ₃ and other PIs	Pleckstrin N-Terminal PtdIns4,5P ₂ 13.4 μ M
Group II	PtdIns3,4,5P ₃ and Ins3,4,5P ₃ with high specificity and affinity	Tiam1 N-Terminal PtdIns3,4,5P ₃ < 1 μ M
Group III	binds PtdIns3,4,5P ₃ and I-1,3,4,5-P	Akt PtdIns3,4P ₂ 570 nM PtdIns3,4,5P ₃ 400 nM
Group IV	no binding or low affinity	

It has not yet been established whether the PH domains from all GEFs are involved in the binding of phospholipids. The charge distribution in DH-PH pairings shows that approximately 50 % have the positive charged residues that are likely to be required for lipid interaction. Indeed in an analysis of PH domains which are negatively charged around the canonical lipid binding site, 5 from 7 were paired with a DH domain and are unlikely to bind acidic phospholipids (Blomberg & Nilges, 1997). In support of this notion Olson *et al.* have shown that the negatively charged PH domain from the Rho-specific GEF Lbc is responsible for localisation of the protein to actin fibres rather to any cellular membrane.

Thus, it has been shown that the PH domain of Dbp-family GEFs can play a regulatory role on exchange activity and that this regulation can be modulated by the binding of PIs. To date most PH domains analysed have specificity for either PtdIns4,5P₂ or PtdIns3,4,5P₃.

To date few PH domains have been described which bind to PtdIns 3,5- P₂. The lipid is synthesised by the sequential action of PI3 and PI-3-P5 kinases. Its physiological

role remains unclear although its synthesis is activated in a PI3 kinase dependent manner in osmotically stressed yeast (Dove *et al.*, 1997). Only β -centaurin, a member of the Arf-GAP family, has been shown to bind solely to PtdIns 3,5- P₂ (Dowler *et al.*, 2000). This family consists of 10 PH domain containing members with differing phospholipid binding profiles, the functional relevance of which remain unclear.

Moreover, the lipid binding specificity of the DH-PH tandem domain from the Rho-GEF Ect2 has not previously been demonstrated.

10 SUMMARY OF THE INVENTION

The present invention describes the isolation of the DH-PH tandem domain from Ect 2 and demonstrates the specificity of this domain for different PIs.

15 Accordingly in one aspect of the invention, there is provided an isolated DH-PH tandem domain derived from the Ect 2 sequence and having the nucleotide sequence as set out in DNA sequence 1 (SEQ ID NO:1). Preferably, the DNA sequence encodes the amino acid sequence set out as amino acid sequence 1 (SEQ ID NO:3). In another embodiment, the isolated DH-PH sequence is part of an expression construct such as
20 that set out in DNA sequence 1 (SEQ ID NO:2) encoding a polypeptide with the amino acid sequence set out as amino acid sequence 2 (SEQ ID NO:4).

In another aspect of the invention there is provided a method of screening for agents that modulate the interaction of the Ect 2 PH domain with PIs, comprising incubating
25 the Ect 2 PH domain polypeptide and said PIs with a candidate agent under conditions conducive for binding and determining whether said candidate agent modulates the binding of the Ect 2 PH domain with the PIs.

By "Ect 2 PH domain" is meant the region of the Ect 2 polypeptide having an amino
30 acid sequence corresponding to the Pleckstrin homology domain as set out in amino acids 644 to 763 of the Ect 2 polypeptide, or a fragment, derivative or homologous sequence thereof. Thus, the phrase "Ect 2 PH domain" is used to described both the

polypeptide sequence and the nucleotide sequence which encodes the polypeptide sequence such as the nucleotide sequence set out in DNA sequence 1 (SEQ ID NO:1). Suitably said Ect 2 PH domain is provided as a folded polypeptide having its natural conformation. In a preferred embodiment, the Ect 2 PH domain is provided as part of the DH-PH tandem domain as described herein. Thus, in a particularly preferred
5 embodiment, the PH domain is provided as part of the construct having a sequence as set out in DNA sequence 1 (SEQ ID NO:1).

In one embodiment, the PI is a PI having a phosphate group at the 3 and/or 5 position
10 of the inositol ring. Suitably PIs include PtdIns 3- P, PtdIns 5- P and PtdIns 3,5- P₂.

In a preferred embodiment, said agent is an antibody, a small organic molecule or an antisense oligomer.

15 In another aspect there is provided a method of identifying an agent that modulates the activity of Ect 2, the method comprising:

- (a) providing a sample containing a polypeptide comprising an Ect 2 PH domain, and a candidate agent;
- (b) measuring the binding of the polypeptide comprising an Ect 2 PH domain to
20 the candidate agent in the sample; and
- (c) comparing the binding of the polypeptide comprising an Ect 2 PH domain to the candidate agent in the sample with the binding of the polypeptide comprising an Ect 2 PH domain to a control agent, wherein the control agent is known to not bind to the polypeptide comprising an Ect 2 PH domain;

25 wherein an increase in the binding of the polypeptide comprising an Ect 2 PH domain to the candidate agent in the sample relative to the binding of the polypeptide comprising an Ect 2 PH domain to the control agent indicates that the candidate agent modulates the cell cycle function of Ect 2.

30 In one embodiment, the activity of Ect 2 is the lipid binding activity. Suitably, the activity of Ect 2 is its cell cycle activity.

The Ect 2 PH domain is demonstrated herein to have specificity for binding to PIs having a phosphate group at the 3 and/or 5 positions. A phosphate group at the 4 position appears to inhibit binding of the PI to Ect 2 even if the 3 and/or 5 positions are phosphorylated.

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Accordingly in another aspect of the invention there is provided the use of a polypeptide capable of binding to PI having a 3 and/or 5 phosphate group but not capable of binding to a PIs having a 4 phosphate group in a screening method for identifying a compound suitable for modulating signalling by a PIs having a 3 and/or 5 phosphate group.

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Suitably, the polypeptide comprises an Ect 2 PH domain. In one embodiment, the Ect 2 PH domain is in the form of a DH-PH tandem domain. In particular the Ect 2 PH domain is provided as a construct having the sequence as set out herein.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a SDS_PAGE and visualised by either coomassie staining (left panels) or by western blotting (right panel). (a) shows expression of Ect2DH, Lane 1: IPTG induction t = 0, Lane 2: IPTG induction t = + 1 hour, Lane 3: IPTG induction t = + 2 hours, Lane 4: IPTG induction t = + 3 hours; (b) shows Refolded Ect2DH, Lanes 1 - 4 show 4 different loadings of refolded Ect2DH

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Figure 2 shows the results of a phospholipid binding assay.

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DNA sequence 1 (SEQ ID NO:1) is the cDNA molecule encoding the DH-PH domains of Ect 2.

DNA sequence 2 (SEQ ID NO:2) is the cDNA molecule construction expressed.

30

AA sequence 1 (SEQ ID NO:3) is the amino acid sequence corresponding to DNA sequence 1 (SEQ ID NO:1).

AA sequence 2 (SEQ ID NO:4) is the amino acid sequence corresponding to DNA sequence 2 (SEQ ID NO:2).

5 DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and
10 biochemistry). Standard techniques are used for molecular, genetic and biochemical methods. See, generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.; as well as Guthrie *et al.*, Guide to Yeast Genetics and Molecular Biology,
15 Methods in Enzymology, Vol. 194, Academic Press, Inc., (1991), PCR Protocols: A Guide to Methods and Applications (Innis, *et al.* 1990. Academic Press, San Diego, Calif.), McPherson *et al.*, PCR Volume 1, Oxford University Press, (1991), Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed. (R. I. Freshney. 1987. Liss, Inc. New York, N.Y.), and Gene Transfer and Expression Protocols, pp. 109-128, ed. E. J.
20 Murray, The Humana Press Inc., Clifton, N.J.). These documents are incorporated herein by reference.

In the context of the present invention the term Ect 2 PH domain also includes within its scope, variants, derivatives and fragments thereof, in as far as they possess the
25 requisite ability to bind PIs with a specificity for those compounds having 3 and/or 5 phosphates.

The terms "variant" or "derivative" in relation to the Ect 2 PH domain polypeptide includes any substitution of, variation of, modification of, replacement of, deletion of
30 or addition of one (or more) amino acids from or to the polypeptide sequence of the Ect 2 PH domain. Preferably, nucleic acids encoding the Ect 2 PH domain are understood to comprise variants or derivatives thereof.

Natural variants of the Ect 2 PH domain are likely to comprise conservative amino acid substitutions. Conservative substitutions may be defined, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

Suitable fragments of the Ect 2 PH domain will be at least about 5, e.g. 10, 12, 15 or 20 amino acids in length. They may also be less than 100, 75 or 50 amino acids in length. They may contain one or more (e.g. 5, 10, 15, or 20) substitutions, deletions or insertions, including conserved substitutions.

Preferred protein derivatives or fragments of the Ect 2 PH domain share at least 80% sequence identity or similarity, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, and most preferably 97% or 100% sequence identity or similarity with a contiguous stretch of the PH domain of Ect 2 as set out at amino acids 644 to 763 of the full length sequence and, in some cases, the entire length of the sequence corresponding to amino acids 644 to 763 of the full length sequence. Preferred Ect 2 PH domains further comprise the amino acids corresponding to amino acids 450-648 (RHOGF domain) of the Ect 2 full amino acid sequence or fragments or derivatives thereof. These domains may be identified using the pfam program (Bateman *et al.*, Nucleic Acids Res. (1999) 27:260-262; <http://pfam.wustl.edu/>), which also contains the detailed description of each domain (RHOGF domain: PF0062 I; PH domain: PFOO 169).

The fragment or derivative of the Ect2 PH domain is preferably “functionally active” meaning that it exhibits one or more functional activities associated with a full-length, wild-type Ect2 PH domain comprising the amino acid sequence of amino acids 644 to
5 763.

As one example, a fragment or derivative may have antigenicity such that it can be used in immunoassays, for immunization, for modulation of Ect2 activity, *etc.* as discussed further below regarding generation of antibodies to Ect2 proteins.
10 Preferably, a functionally active Ect2 PH domain fragment or derivative is one that displays one or more biological activities, such as signaling activity or binding to PIs with a specificity to those having a 3 or 5 phosphate.

The term “Ect2 nucleic acid” refers to a DNA or RNA molecule that encodes an Ect2
15 polypeptide. Preferably, the Ect2 PH domain polypeptide or nucleic acid or fragment thereof is from a human (e.g. as set out in DNA sequence 1 (SEQ ID NO:1) herein), but it can be an ortholog or derivative thereof, preferably with at least 70%, 80%, 85%, 90%, or 95% sequence identity to DNA sequence 1 (SEQ ID NO:1). Orthologs can be identified by BLAST analysis using DNA sequence 1 (SEQ ID NO:1), using methods
20 known in the art (Huynen MA and Fork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10: 1204-1210).

Isolation, Production, and Expression of Ect2 Nucleic Acids and Polynucleotides

A wide variety of methods are available for obtaining Ect2 PH domain polypeptides.
25 In general, the intended use for the polypeptide will dictate the particulars of expression, production, and purification methods. For instance, production of polypeptides for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of polypeptides for antibody generation may require structural integrity of particular
30 epitopes. Expression of polypeptides to be purified for screening or antibody production may require the addition of specific tags (i.e., generation of fusion proteins). Techniques for the expression, production, and purification of proteins are

well known in the art; any suitable means therefor may be used (e.g., Higgins SI and Hames ED (eds.) Protein Expression: A Practical Approach, Oxford University Press Inc., New York 1999; Stanbury PF et al., Principles of Fermentation Technology, 2nd edition, Elsevier Science, New York, 1995; Doonan S (ed) Protein Purification
5 Protocols, Humana Press, New Jersey, 1996; Coligan JE et al, Current Protocols in Protein Science (eds.), 1999, John Wiley & Sons, New York; U.S. Pat. No. 6,165,992). The nucleotide sequence encoding an Ect2 PH domain polypeptide can be inserted into any appropriate vector for expression of the inserted protein-coding sequence. The necessary transcriptional and translational signals, including promoter/enhancer
10 element, can derive from the native ect2 gene and/or its flanking regions or can be heterologous.

The ect2 gene may be expressed in prokaryotic or eukaryotic cells, the method of choice depends on the intended use of the protein. In particular, eukaryotic systems are
15 particularly useful when native folding and post translational modifications are required. Preferred prokaryotic cells include *Escherichia coli* and *Bacillus subtilis*. Preferred eukaryotic cells include mammalian cells (such as human, mouse, monkey or Chinese hamster ovary cells), yeast cells (such as *Pichia* and *Saccharomyces* species) and insect cells (such as *Drosophila* and various lepidopteran-cell lines, e.g. Sf9 cells).
20 Cell extracts or supernatants may be purified in order to isolate the Ect2 polypeptide. Preferred purification techniques include HPLC, size exclusion chromatography, cation and anion exchange chromatography, reverse phase chromatography, affinity chromatography and other protein purification techniques known to those skilled in the art.

25
The Ect2 PH domain polypeptide may be optionally expressed as a fusion or chimeric product, joined via a peptide bond to a heterologous protein sequence. For example, to facilitate detection and/or purification of Ect2-derived polypeptide, the Ect2 expression vector construct may contain one or more antibody epitope coding
30 sequences introduced at the N-terminus, C-terminus of the Ect2 coding region and/or at any position within the gene sequence. Suitable sequences include the Myc epitope, HA epitope, FLAG epitope or polyhistidine epitope (see, e.g Harlow and Lane (1988))

Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory). As another example, the Ect2 PH domain polypeptide may be expressed as a fusion protein joined to a transcriptional reporter such as GFP or luciferase. A chimeric protein can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other in the proper coding frame using standard methods and
5 expressing the chimeric product. A chimeric protein may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

10 Ect2-Modulating Agents

The invention provides methods to identify agents that interact with and/or modulate the function of Ect2 and/or the p21 pathway through interaction with the PH domain and thus through modulation of binding to PIs and, particularly those PIs having a 3 and/or 5 phosphate. Such agents are useful in a variety of diagnostic and therapeutic
15 applications associated with diseases or disorders involving a defective p21 pathway, cell cycle and inositol signalling as well as in further analysis of the Ect2 protein and its contribution to the cell cycle and the p21 pathway.

In a preferred embodiment, Ect2-modulating agents inhibit or enhance Ect2 activity or
20 otherwise affect normal Ect2 function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In a further preferred embodiment, the candidate inositol signalling pathway-modulating agent specifically modulates the function of the Ect2. The phrases 'specific modulating agent', 'specifically modulates', etc., are used herein to refer to modulating agents that
25 directly bind to the Ect2 polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter the function of the Ect2. The term also encompasses modulating agents that alter the interaction the Ect2 with a binding partner or substrate (*e.g.* by binding to a binding partner of an Ect2, or to a protein/binding partner complex, and inhibiting function).

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Preferred Ect2-modulating agents include small molecule chemical agents, Ect2-interacting proteins, including antibodies and other biotherapeutics, and nucleic acid

modulators, including antisense oligomers and RNA. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Methods of formulating biotherapeutic agents are described in detail in
5 U.S. Pat. No. 6,146,628. Techniques for formulation and administration of compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19th edition.

Small Molecule Modulators

10 Small molecule modulators are typically organic, non-peptide molecules, having a molecular weight less than 10,000, preferably less than 5,000, more preferably less than 1,000, and most preferably less than 500. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Small molecule modulators may be rationally designed based on
15 known structural properties, for example, their ability to bind to PIs having a 3 and/or 5 phosphate group, or other properties discerned using methods described above. Structures of Ect2 in complex with the partner G-protein (RhoA/Rac/CDC42) show the details of protein-protein interactions required for the GEF activity and can be used to aid in the rational design of small-molecule compounds that modulate the
20 mechanics of these interactions, thereby disrupting the GEF functionality. Structures of Ect2 polypeptides in complex with small-molecule ligands which serve to modulate the GEF activity delineate the portions of the Ect2 molecule which are either directly involved in the catalytic active site or which exert an allosteric effect on the active site, thereby modulating the GEF activity. Small molecule modulators may also be
25 identified by screening compound libraries.

Alternative small molecule modulators include natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for Ect2—modulating activity. Methods for generating
30 and obtaining compounds are well known in the art (Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 15 1:1947-1948).

Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with the defective cell cycle or inositol signaling. The activity of candidate small molecule modulating agents may be improved-several-fold through iterative secondary functional validation, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and rescreened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

Protein Modulators

A protein which interacts with the Ect2 PH domain may be endogenous, *i.e.* one that normally interacts genetically or biochemically with Ect2. Ect2-modulators include dominant negative forms of Ect2-interacting proteins and of Ect2 proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous Ect2-interacting (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry offers alternative preferred methods for the elucidation of protein complexes (reviewed in, *e.g.*, Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR, Trends Genet (2000) 16:5-8).

An Ect2-interacting protein may be exogenous protein, such as an Ect2-specific antibody or a T-cell antigen receptor (see. *e.g.*, Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory). Ect2 antibodies are further discussed below.

In one preferred embodiment, an Ect2—interacting protein specifically binds an Ect2 PH domain protein. In an alternative preferred embodiment an Ect2—modulating

agent binds an Ect2 substrate, binding partner, or cofactor. In certain applications when Ect2-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the Ect2 protein may be assayed by various known methods, including binding equilibrium constants
5 (usually at least about 10^7M) and immunogenic properties. For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

Specific antibodies

In a preferred embodiment, the Ect2-interacting protein is an antibody. Antibodies that
10 specifically bind Ect2 polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian Ect2 polypeptide, and more preferably, a human Ect2. In a particularly preferred embodiment, the antibody specifically binds to the Ect 2 PH domain.

15 Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab Fragments, F(ab')₂ fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Monoclonal antibodies with affinities of 10^8M^{-1} preferably 10^9M^{-1} to 10^{10}M^{-1} , or stronger can be made by standard procedures as
20 described (Harlow and Lane, Antibodies: A Laboratory Manual, CSH Laboratory (1988); Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against extracts of cells that express Ect2 or from substantially purified Ect2 or fragments thereof. If Ect2 fragments are used, they
25 preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of an Ect2 protein. Suitably, the Ect 2 fragments comprise the PH domain. In a particular embodiment Ect2-specific antigens and/or immunogens are coupled to carrier proteins that stimulate the immune response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier,
30 and the conjugate is emulsified in Freund's complete adjuvant. which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols.

The presence of Ect2-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding Ect2 polypeptides. Other assays, such as radioimmunoassays or
5 fluorescent assays might also be used.

Chimeric antibodies specific to Ect2 polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the
10 antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al., Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a
15 form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., 3. M. Harlan., Blood (1994) 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., Nature (1988) 323:323-327). Humanized antibodies contain approx 10% murine sequences and approx 90% human
20 sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C., Nature (1991) 35 1:501-501; Morrison SL., Ann. Rev. Immun. (1992) 10:239-265). Humanized antibodies and methods of their production are well-known in the art (U.S. Pat Nos. 5,530,101; 5,585,089; 5,693,762, and 6,180,370).

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Ect2-specific single chain antibodies, which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced (U.S. Pat-. No. 4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA (1988) 85:5879-5883;
30 and Ward et al., Nature (1989) 334:544-546).

Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Ruse et al., Science (1989) 246:1275-1281).

5 The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells that express the targeted protein (Menard S, et al., Int J. Biol Markers (1989)4: 131-134). A wide variety of labels and conjugation techniques are
10 known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like-(U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also,
15 recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic proteins may be delivered and reach-their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. NO. 6,086,900).

When used therapeutically in a patient, the antibodies of the subject invention are
20 typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg to about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension,
25 emulsion) in association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance
30 isotonicity and chemical stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml

to about 10 mg/ml. Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; W00073469).

Nucleic Acid Modulators

- 5 Other preferred Ect2-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit Ect2 activity.

- Preferred antisense oligomers interfere with the function of Ect2 nucleic acids, such as
10 DNA replication, transcription, Ect2 RNA translocation, translation of protein from the Ect2 RNA, RNA splicing, and any catalytic activity in which the Ect2 RNA.

- Alternative preferred Ect2-modulating agents are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific, post-
15 transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and mammals are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001);
20 Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101,25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); W00129058; W09932619, and Elbashir SM, et
25 al., 2001 Nature 411:494-498),

Assay Systems

- The invention provides assay systems for identifying specific modulators of Ect2 activity. In general, primary assays are used to identify or confirm a modulator's
30 specific biochemical or molecular effect with respect to the Ect2 nucleic acid or protein or Ect 2 PH domain. In general, secondary assays further assess the activity of

an Ect2-modulating agent identified by a primary assay and may confirm that the modulating agent affects Ect2 in a manner relevant to cell cycle regulation.

The pleckstrin homology (PH) domain comprises 2 perpendicular anti-parallel beta sheets and a C-terminal amphipathic helix. A large variability in the connecting loops has been reported. The reported functions for the PH domain include binding of the beta/gamma subunit of heteromeric G-proteins, PtdIns4,5P₂ binding, phospho-Ser/Thr binding and membrane interaction.

10 The RhoGEF Domain (DH or Dbl domain) comprises 11 alpha helices folded into a flattened, elongated alpha-helix bundle. Its role is thought to be to accelerate dissociation of GDP bound to Rho family small GTPases and to promote GTP binding, thus activating signalling.

15 Suitable assays for detecting the ability of a small molecule to inhibit GEF activity include incubating an Ect 2 -derived polypeptide with Rho A in the presence of ³H GDP and detecting the retention of ³H in the presence or absence of the candidate small molecule.

20 Primary Assays

The type of modulator tested generally determines the type of primary assay.

For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam OS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying references). As used herein the term "cell-based" refers to assays using live cells, dead cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. Cell-based screening assays usually require systems for recombinant expression of the Ect2 PH domain and any auxiliary proteins demanded by the particular assay. The term 'cell free' encompasses assays using substantially purified protein (either endogenous or recombinantly produced), partially purified

cellular extracts, or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding), transcriptional activity (*e.g.*, using a reporter gene, enzymatic activity (*e.g.* via a property of the substrate), activity of second messengers, immunogenicity and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide range of detection methods including fluorescent (Klebe C, et al., *Biochemistry* (1995) 34:12543-12552), radioactive (Hart M, et al., *Nature* (1991) 354:311-314), colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular molecular event detected, often in high throughput screening (HTS) formats (for example, see HertzbergRP, and Pope AJ, *Current Opinion in Chemical Biology* (2000) 4:445-451).

Assays for binding agents include screens for compounds that modulate Ect2 interaction with a natural Ect2 binding target. The Ect2 polypeptide used in such assays may be fused to another polypeptide such as a peptide tag for detection or anchoring, etc. In a particular embodiment, the binding target is RhoA, RhoC, Rac, or Cdc42, or portion thereof that provides binding affinity and avidity to the subject Ect2 polypeptide conveniently measurable in the assay and preferably comparable to the intact RhoA, RhoC, Rae, or Cdc42. The Ect2 and binding target are incubated in the presence and absence (*i.e.* control) of a candidate Ect2 modulating agent under conditions whereby, but for the presence of the candidate modulating agent, the Ect2 polypeptide specifically binds the cellular binding target, portion or analog with a reference binding affinity. After incubation, the agent-biased binding between the Ect2 polypeptide and one or more binding targets is detected by any of a variety of methods depending on the nature of the product and other assay components, such as through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirect detection with antibody conjugates, etc. A difference in the binding affinity of Ect2 to the target in the absence of the agent, as compared with the binding affinity in the presence of the agent indicates that the agent modulates the binding of the Ect2 to the Ect2 binding target. A difference, as used herein, is statistically significant and preferably represents at least a 50%, preferably at least 60%, more preferably 75%, and most preferably a 90% difference.

Other preferred assay formats use fluorescence technologies, including fluorescence polarization, time-resolved fluorescence, and fluorescence resonance energy transfer. These systems offer means to monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.*, Selvin PR, Nat Struct Biol (2000) 7:730-4; Fernandes PB, Curr Opin Chem Biol (1998) 2:597-603; Hertzberg RP and Pope AJ, Curr Opin Chem Biol (2000) 4:445-451).

For antibody modulators, appropriate primary assays test the antibody's specificity for and affinity to the Ect2 PH domain protein. Methods for testing antibody specificity and affinity are well known in the art. Alternatively or additionally, primary assays for antibody modulators may comprise the screening assays described above, used to detect the Ect2 modulators specific activity.

Secondary validation can use essentially the same assays used to functionally validate the participation of ect2 PH domain in an inositol-signalling related pathway. Secondary validation assays generally compare like populations of cells (*e.g.*, two pools of wild type cells) in the presence and absence of the candidate modulator.

In another embodiment, secondary validation may use the same assays used for high throughput screening. These methods can confirm the activity of a modulator not identified through high throughput screening, such as an antibody or an antisense oligonucleotide modulator, or can confirm the activity of a small molecule modulator identified using a different high throughput screening assay. These assays may also be used to confirm the specificity of a candidate modulator.

Additionally, the modulator is assayed for its effectiveness on the Ect2 in a cell cycle related manner. Such assays include cell cycle, apoptosis, proliferation. and hypoxic induction assays, among others, as described above.

Therapeutic and diagnostic applications

When used for anti-tumor therapy in a patient, Ect2 modulating agents are administered to the patient in therapeutically effective amounts that eliminate or reduce the patient's tumor burden. They will normally be administered parenterally, when possible at the target cell site, or intravenously. The dose and dosage regimen will depend upon the nature of the cancer (primary or metastatic), its population, the target site, the characteristics of the particular immunotoxin (when used), e.g., its therapeutic index, whether the agent is administered in combination with other therapeutic agents, and the patient's history. The amount of agent administered will typically be in the range of approximately 0.1 - 10 mg/kg of patient weight.

For parenteral administration, the agents will be formulated in a unit dosage injectable or inhalable (solution, suspension, emulsion) form in association with a pharmaceutically acceptable vehicle, typically in a concentration of about 1 – 10 mg/ml.

Antibodies that specifically bind Ect2 may be used for the diagnosis of conditions or diseases characterized by expression of Ect2, or in assays to monitor patients being treated with Ect2 modulating agents. Diagnostic assays for Ect2 include methods which utilize the antibody and a label to detect Ect2 in human body fluids or extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule.

Diagnosis of conditions characterized by expression of Ect2 may also be accomplished by any of a variety of methods such as Northern or TaqMan® analysis (discussed *supra*) to measure expression of Ect2 in patient samples.

- 5 The present invention will now be described with reference but not limitation to the following examples.

EXAMPLES

10 Methods

Ect2 Cloning and Expression

- The cDNA encoding the DH-PH domains (DNA sequence 1 (SEQ ID NO: 1), with the corresponding amino acid sequence given as AA sequence 1 (SEQ ID NO:3)) of Ect2
15 was cloned into the EcoR1 site in pENTR-3c vector (GIBCO). This sequence was then transferred using a recombination reaction to pDEST10 (GIBCO) giving a construct for expression of a 434 aa protein with estimated molecular weight of 49.8 kD containing the DH domain (aa 39 – 235) and the PH domain (aa 269 – 388) from Ect2 as well as an N-terminal his-tag (aa 5 – 10) for purification. The sequence of the
20 final expression construct is given in DNA sequence 2 (SEQ ID NO:2).

DNA sequence 1 (SEQ ID NO:1)

- ccagttccttcaaagcagtcagcaaggtggcaagttgcaaaagagctttatcaaactgaaagtaattatgttaatatattggcaa
caattattcagttatttcaagtaccattggaagaggaaggacaacgtgggtggacctatcctgcaccagaggagattaagacta
25 ttttggtagcatcccagatatcttgatgtacacactaagataaaggatgatcttgaagaccttatagttaattgggatgagagc
aaaagcattgggtgacatttttctgaaatattcaaaagatttggtaaaaacctaccctcccttggtaaactctttgaaatgagcaag
gaaacaattattaaatgtgaaaaacagaaaccaagatttcatgcttttctcaagataaaccaagcaaaaccagaatgtggacg
gcagagccttggtgaacttctatccgaccagtacagagggttaccagtggtgcattacttttaaatgatcttaagaagcatacag
ctgatgaaaatccagacaaaagcactttagaaaaagctattggatcactgaagggaagtaatgacgcatattaatgaggataag
30 agaaaaacagaagctcaaaagcaaattttgatgttggttatgaagtagatggatgccagctaatttttatcttctcaccgaag
cttagtacagcgggttgaaacaatttctctaggtgagcaccctgtgacagaggagaacaagtaactcttctcttcaatgat
tgcctagagatagcaagaaaacggcacaagggttattggcacttttaggagtcctcatggccaaacccgacccccagcttctct

taagcatattcacctaatagcctctttctcagattaagaaggtattggacataagagagacagaagattgccataatgctttgcct
tgcttgtagggccaccaacagagcaggcaaatgtgctactcagttccagatgacatcagatgaactccaaaagaaaactg
gctaaagatgctgtgtcgacatgtagctaacaccatttgtaaa

5 **DNA sequence 2 (SEQ ID NO:2)**

atgtcgtactaccatcaccatcacctcgaatcaacaagttgtacaaaaagcaggctctttaaggaaccaattcag
tcgactggatccggtaccgaattgcccttccagttcctcaaagcagtcagcaaggtggcaagttgcaaaagagctttatca
aactgaaagtaattatgttaatatattggcaacaattattcagttattcaagtaccattggaagaggaaggacaacgtggtgga
10 cctatccttgaccagaggagattaagactattttggtagcatccagatatctttgatgtacacactaagataaaggatgatct
tgaagaccttatagtaattgggatgagagcaaaagcattggtgacattttctgaaatattcaaaagatttggtaaaaacctacc
ctcccttgtaaactctttgaaatgagcaaggaaacaattattaaatgtgaaaaacagaaaccaagatttcagcttttctcaag
ataaaccaagcaaaaccagaatgtggacggcagagcctgttgaacttctatccgaccagtacagaggttaccagtggtgc
attacttttaaatgatcttaagaagcatacagctgatgaaaatccagacaaaagcacttagaaaaagctattggatcactgaag
15 gaagtaatgacgcatattaatgaggataagagaaaaacagaagctcaaaagcaaatttttagtggtttatgaagtagatgga
tgccagctaattctttatcttctcaccgaagcttagtacagcgggttgaaacaatttctctaggtgagcaccctgtgacagag
gagaacaagtaactcttctcttcaatgattgcctagagatagcaagaaaacggcacaaggttattggcacttttaggagtc
ctcatggccaaacccgacccccagcttctttaaagcatattcacctaatagcctctttctcagattaagaaggtattggacataag
agagacagaagattgccataatgcttttgccttgcttgtagggccaccaacagagcaggcaaatgtgctactcagttccagat
20 gacatcagatgaactccaaaagaaaactggctaaagatgctgtgtcgacatgtagctaacaccatttgtaaagcaagggcg
aattcgcgccgcactcgagatatctagaccagcttctgtacaaagtgggtgattcgaggctgctaacaagcccgaag
gaagctgagttggctgctgccaccgctgagcaataactag

AA sequence 1 (SEQ ID NO:3)

25 Pvpksqsarwqvakelyqtesnyvnilatiiqlfvpleeegqrggpilapeeiktifgsipdifdvhtkikddledlivnw
desksigdiflkyskdllvktppfvnffemsketiikcekqkprfhafkinqakpecgrqslvellirpvqrlpsvallnd
lkkhtadenpdkstlekaigslkevmthinedkrkteaqkqifdvvyevdgcpanllsshrslvqrvetislgehpdrge
qvtilflndcleiarkrhkvigtfrsphqtrppaslkhhlmpslqikkvldirededchnafallvrppteqanvllsfqmt
sdelpkenwlkmlcrhvantick

30

AA sequence 2 (SEQ ID NO:4)

msyyhhhhhhlestsllykkagslkepiqstgsgtefalvpvpsqsarwqvakelyqtesnyvnilatiiqlfqvpleeeg
 qrggpilapeeiktifgsipdifdvhtkikddledlivnwdesksigdiflkyskdldvktypfvnffemsketiikcekqk
 prfhafkinqakpecgrqslvellirpvqrlpsvallndlkkhtadenpdkstlekaigslkevmtthinedkrkteaqkq
 ifdvvyevdgcpanllsshrslvqrvetislgehpcdrgeqvltflndcleiarkrhkvigtfrsphgqtrppaslkhihlm
 5 plsqikkvldiredchnafallvrppteqanvllsfqmtsdelphenwklmcrhvantickaransrphsryldpafly
 kvvdsrlltkperklswllpplsnn

Ect2-DH-PH was expressed in Rosetta(DE3)pLysS cells. Cells were grown at 37°C and harvested 3 hours after induction with 1mM IPTG. The inclusion body fraction
 10 was isolated by treating the cell pellet with BugBuster HT protein extraction reagent (Novagen) according to the manufacturer's instructions.

The inclusion body pellet was resolubilized in 50mM TrisHCl pH8.0/ 1mM DTT/ 6M GuHCl at a protein concentration of 0.38mg/ml. The protein was then centrifuged at
 15 15000rpm for 15 min to remove any potential nucleation sites. Refolding of the protein was initiated by rapid dilution (1:11) of the soluble fraction into 'refolding buffer' 50mM TrisHCl pH8.0/ 1mM DTT/ 10% glycerol/ 100mM NaCl. The sample was then filtered through a 0.2µm filter followed by extensive dialysis, overnight at 4°C, against 'refolding buffer' to remove any residual traces of GdnHCl. The protein
 20 was filtered again through a 0.2µm filter followed by concentration using an Amicon Centriprep with a 10kDa cut off. The protein at this stage was judged to be > 80% pure by SDS-PAGE analysis.

Phospholipid Binding Assay

25 To assess the phospholipid binding specificities of the DH-PH domain of Ect2 (Ect2-DH-PH) a lipid overlay assay was performed. Lipid solution (1µl) containing 250 pmoles of phospholipids dissolved in a mixture of chloroform/methanol/water (1:2:0.8) was spotted onto Hybond-C extra membrane and allowed to dry at room temperature. The membrane was blocked by incubation for 60 minutes in TBST (10 mM Tris/Hcl, pH 8.0, 150 mM NaCl, 0.1 % Tween-20) containing 3%(w:v) fatty acid free BSA.
 30 The membrane was then incubated overnight at 4°C in the same solution containing 0.5 mg/ml Ect2-DH-PH. The membrane was then washed extensively with TBST and

incubated for 60 minutes with TBST containing a 1:1000 dilution of anti-6*his antibody (Sigma). The membrane was washed as before with TBST prior to incubation for 60 mins in TBST containing a 1:5000 dilution of an anti-mouse-HRP conjugate. The membrane was then washed as before and the presence of Ect2-DH-
 5 PH was detected by soaking the membrane in ECL reagents (AmershamPharmacia) and exposing the membrane to photographic film (Kodak).

Results

10 Figure 1 shows Ect2DH-PH expression.

Ect2DH-PH was expressed in *E.Coli* (DE3)pLysS as insoluble protein. An inclusion body fraction was prepared and the protein was refolded by denaturation into a buffer containing 6M GdnHCl followed by rapid dilution and dialysis to remove the denaturant. Samples were separated by SDS_PAGE and visualised by either
 15 coomassie staining (left panels) or by western blotting (right panel). Figure 1 (a) shows expression of Ect2DH, Lane 1: IPTG induction t = 0, Lane 2: IPTG induction t = + 1 hour, Lane 3: IPTG induction t = + 2 hours, Lane 4: IPTG induction t = + 3 hours. Figure 1 (b) shows refolded Ect2DH, Lanes 1 - 4 show 4 different loadings of refolded Ect2DH

20

Figure 2 demonstrates the specificity of Ect 2 PIP binding.

- | | |
|------|--|
| 1 | Phosphatidyl choline |
| 2 | Phosphatidyl ethanolamine |
| 3 | Phosphatidyl inositol |
| 25 4 | Phosphatidyl inositol – 3 phosphate (PtdIns3P) |
| 5 | Phosphatidyl inositol – 4 phosphate (PtdIns4P) |
| 6 | Phosphatidyl inositol – 5 phosphate (PtdIns5P) |
| 7 | Phosphatidyl inositol – 3,4 phosphate (PtdIns3,4P ₂) |
| 8 | Phosphatidyl inositol – 3,5 phosphate (PtdIns3,5P ₂) |
| 30 9 | Phosphatidyl inositol – 4,5 phosphate (PtdIns4,5P ₂) |

These results suggest that a PO_3^- at position 3 and/or 5 promotes binding while a PO_3^- at position 4 inhibits binding even if 3 or 5 position phosphorylated.

5

References

Blomberg, N. & Nilges, M., *Folding & Design*, 2, 343 – 355, 1997.

10 Bustelo, X.R., *Mol. Cell Biol.*, 20, 1461 – 1477, 2000.

Das *et al.*, *J. Biol. Chem.*, 275, 15074 – 15081, 2000.

Han *et al.*, *Science*, 279, 558 – 560, 1998.

15

Kavran *et al.*, *J. Bio. Che.*, 273, 30497 – 30508, 1998.

Kawasaki *et al.*, *Science*, 289, 1194 – 1197, 2000.

20 Nimnual *et al.*, *Science*, 279, 560 – 563.

Rameh *et al.*, *J. Biol. Chem.*, 272, 22059 – 22066, 1997.

Russo *et al.*, *J. Biol. Chem.*, 276, 19524 – 19531, 2001.

25

Tatsumoto *et al.*, *J. Cell Biol.*, 147(5), 921 – 927, 1999.

Zheng, Y., *TIBS*, 26(12), 724 – 732, 2001.

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All patents, patent applications, and published references cited herein are hereby incorporated by reference in their entirety. While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those of ordinary skill in the art that various changes in form and details
35 may be made herein without departing from the scope of the invention encompassed by the appended claims.